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Recognition of Mycobacterial Antigens by Sera from Patients with Leprosy

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Mycobacterium leprae **sonic extracts prepared from armadillo-derived bacteria were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) procedures and probed with serum or plasma samples from 20 patients with lepromatous leprosy and 14 healthy endemic controls. Five proteins of 33, 25, 18, 15, and 12 kilodaltons (kDa) were frequently recognized; the 33- and 15-kDa proteins were, respectively, recognized with high intensity by 16 and 13 of the 20 samples from patients with leprosy, whereas only one healthy donor had antibodies that recognized the 15-kDa protein. By the use of *M. leprae*-specific murine monoclonal antibodies it was demonstrated that the 33-, 25-, and 15-kDa antigens were different from those bound by the available murine monoclonal antibodies. The 18- and 12-kDa proteins detected had molecular masses similar to those detected by the corresponding murine monoclonal antibodies. The serum and plasma samples from patients with leprosy were also used to probe Western blots of a soluble extract of *M. tuberculosis*. They recognized, among others, antigens with molecular weights similar to those detected in the *M. leprae* antigenic preparations, although with less intensity and at a lower frequency.**

Leprosy is a chronic granulomatous disease produced by infection with *Mycobacterium leprae*, an intracellular, noncultivable, acid-fast bacillus which affects about 12 million people worldwide. A knowledge of the antigenic composition of the bacterium and the role that these antigens play in the immune response during infection is important for understanding the pathogenesis of the disease. Several *M. leprae* antigens with diverse biochemical compositions have been described by studying the antibody responses from patients across the leprosy spectrum by using a variety of methods (9). One *M. leprae*-specific antigen, phenolic glycolipid I, is currently being used for diagnosis of multibacillary leprosy (18); an enzyme-linked immunosorbent assay has been developed that uses a synthetic disaccharide from phenolic glycolipid I coupled to bovine serum albumin which allows detection of immunoglobulin M (IgM) class antibodies to the phenolic glycolipid I molecule. However, the test is not useful for the diagnosis of tuberculoid leprosy or other forms of paucibacillary disease, since the sensitivity of the assay decreases toward this pole of the leprosy spectrum. Crossed immunoelectrophoresis studies identified more than 20 different antigenic components of *M. leprae* (7), whereas more recently, by the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques, several protein antigens have been recognized (4, 6, 8, 13). Their immunopathogenic role or diagnostic potential remains to be established.

The genes that encode six protein antigens of *M. leprae* (70, 65, 36, 28, 18, and 12 kilodaltons [kDa]) have been cloned by using murine monoclonal antibodies (1, 20). However, these proteins represent a small proportion of the proteins produced by *M. leprae*. Presumably there are other structural and secreted antigens unrecognized by the few available murine monoclonal antibodies but relevant in the immunology of the human disease. The identification, cloning, and characterization of immunodominant antigens will provide reagents for functional studies of immunity, development of monoclonal antibodies, diagnosis in seroepidemiological surveys, and finally, selection of potentially protective antigens in vaccine development. This report describes the identification of *M. leprae* protein antigens with sera from 20 lepromatous patients from Mexico and Guyana by the use of SDS-PAGE analysis and immunoblotting techniques.

MATERIALS AND METHODS

Chemicals. General chemicals were obtained from BDH (Poole, England). Coomassie blue and amido black stains, diaminobenzidine, hydrogen peroxide, goat anti-human peroxidase-conjugated antibodies, protease (type XIV; 5.8 U/mg) from *Streptomyces griseus*, and trypsin (type III; 11,700 α-*N*-benzoyl-L-arginine ethyl ester U/mg) from bovine pancreas were obtained from Sigma Chemical Co., (Poole, England). Goat anti-mouse peroxidase-conjugated antibody was from Bio-Rad Laboratories, Watford, England, and 4-chloro-1-naphthol was from GIBCO-BRL (Paisley, Scotland). The molecular mass standards used were phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin (20 kDa), and lactalbumin (14 kDa) from Pharmacia (Milton Keynes, England).

Patients. Twenty patients (10 male, 10 female) with lepromatous leprosy (LL) were included in this study. Fourteen patients were from Mexico, and six were from Guyana, with a mean age of 36 ± 13 years. Blood samples and clinical information were obtained with the consent of patients and ethics committee approval. The diagnoses of the patients were established by specialized medical staff by using clinical and histopathological criteria based on the Ridley and Jopling classification (16). All of the patients were receiving multiple drug therapy as recommended by the World Health Organization (WHO). Eleven had a history of erythema

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nodosum leprosum, but none were in reaction when blood samples were collected. Most of the patients had chronic LL, with a mean disease duration of 12 years. No other relevant medical conditions were apparent at the time of the study. The Mexican patients had not received *Mycobacterium bovis* BCG vaccination; the BCG status of patients from Guyana was not known.

Controls. Nine male and five female healthy individuals (eight from Mexico and six from Guyana) with a mean age of 37 ± 13 years were included in the study as control subjects. Five of eight Mexican subjects were BCG vaccinated; the BCG status of those from Guyana was not known.

Collection of serum and plasma samples. A peripheral blood sample of 20 ml was obtained from each Mexican patient and control subject without the use of heparin. Serum samples were collected and portions were frozen at −70°C until used. Plasma samples from Guyanese patients and controls (diluted 1:3 with RPMI culture medium) were stored at −20°C.

Antigenic preparations. Three different batches of *M. leprae* sonic extract (CD67, CD75, and CD99) prepared from infected armadillo tissue were kindly provided by R. J. W. Rees from the National Institute for Medical Research, London, England. These sonic extracts had been prepared by the Draper protocol [*WHO 1980, Report on the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy (Immlep)*; WHO document TDR/IMM-LEP-SWG (5) 80.3 annex 4, p. 23]. A soluble extract from *M. tuberculosis* H37RV (ATCC 27294) was kindly provided by Saroj K. Young and George K. Varghese (London School of Hygiene and Tropical Medicine).

Immunoblotting. The mycobacterial antigenic preparations (10 μg per lane) were fractionated by SDS-PAGE (14) in a 12% resolving gel (monomer-dimer ratio, 30:1) with minigels (8 by 5 cm by 0.75 mm thick) on a vertical double-slab unit (Hoeffer Scientific Instruments, San Francisco, Calif.).

Proteins from the gels were electrophoretically transferred to nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany) (17); the transfer was performed at 50 V for 1 h. After blotting, the gels were stained with 0.125% Coomassie blue R-250 solution to ensure that complete transfer occurred. The filter was briefly stained in amido black, cut into individual tracks, and washed in distilled water. The strips were washed in phosphate-buffered saline (PBS; pH 7.2)–0.2% Triton X-100 solution, placed on a glass plate, wrapped in cling film, and stored overnight at 4°C.

Nitrocellulose strips were transferred individually to six-well plastic plates (each well was 3 cm in diameter and had a 15-ml capacity; Costar, Cambridge, Mass.). After additional protein-binding sites were blocked for 1 h with 1% bovine serum albumin in PBS–Triton X-100 solution, the antigen strips were incubated for 1 h with 10 ml of serum or plasma diluted in PBS–Triton X-100–bovine serum albumin solution (1:150 for IgG experiments and 1:50 for IgM experiments).

The strips were individually washed three times for 10 min each time with PBS–Triton X-100 solution and incubated for 1 h with the second antibody solution (goat anti-human IgG or IgM peroxidase conjugates diluted in PBS–Triton X-100–bovine serum albumin solution). For experiments in which murine monoclonal antibodies were used as the first antibody, goat anti-mouse IgG peroxidase conjugate was used as the second layer. After incubation with the second antibody, the blots were washed three times with PBS–Triton X-100 solution.

The substrate used was a combination of 4-chloro-1-naphthol and diaminobenzidine, which is more effective than either substrate alone, having the sensitivity of diaminobenzidine but with a purple-black color (Paul Young, personal communication). Diaminobenzidine (3.6 mg) was dissolved in 30 ml of citrate buffer (pH 5), and 4-chloro-1-naphthol (6 ml of a 3-mg/ml solution in methanol) was added. Immediately before use, 24 μl of 30% H_2O_2 was added. Color development was rapid and was stopped by rinsing the filters in tap water, followed by a brief (30-s) rinse in 1 mM sulfuric acid. The filters were washed thoroughly with distilled water, air dried, and photographed.

The presence and intensity of bands on the blots were assessed independently by three different individuals and scored as moderate (+) or high (++) intensity; attention was given to single major bands as visualized by the naked eye. The molecular masses of the bands were calculated from the markers on each filter. To accurately compare bands on tracks from different gels, selected samples used in different experiments were probed together.

Protease digestion of *M. leprae* sonic extracts. *M. leprae* sonic extract CD99 was subjected to digestion with either trypsin or *S. griseus* protease. A 1-mg/ml stock solution was prepared of each enzyme (trypsin was diluted in 0.1 mM HCl, whereas *S. griseus* protease was diluted in 0.1 mM Tris hydrochloride [pH 8]–1 mM $CaCl_2$). Two microliters of trypsin or 4 μl of an *S. griseus* protease stock solution was added to 50-μg sonic extract samples. The mixture of *M. leprae* sonic extract plus either enzyme was incubated at 37°C for 1 h. Enzyme-treated and whole sonic extract samples were subjected to SDS-PAGE analysis in duplicate minigels. One-half of the first gel was stained with Coomassie blue, and the other half was stained with periodic acid-Schiff reagent. The second gel was blotted onto a nitrocellulose filter as described in the previous section; enzyme-treated and whole sonic extract antigenic strips were probed with a selected LL serum sample.

Murine monoclonal antibodies. The following five *M. leprae*-specific murine monoclonal antibodies were used: L12 (65 kDa), F47-9 (36 kDa), SA1 B11 H (28 kDa), L5 (18 kDa), and ML06 (12 kDa) (2, 3, 11, 12, 19). F47-9 and SA1 B11 H were used at a 1:1,000 dilution, L12 and L5 were used at a 1:10,000 dilution, and ML06 was used at a 1:500 dilution. ML06 was generously provided by J. Ivanyi, Hammersmith Hospital, London, England, and the other antibodies were provided by the WHO.

RESULTS

SDS-PAGE and Western blotting (immunoblotting) of *M. leprae* sonic extracts. *M. leprae* sonic extracts were fractionated by SDS-PAGE and transferred to nitrocellulose filter membranes. To determine the profile of the electrophoresed proteins as well as the efficiency of the blotting procedure, filters were stained with amido black. More than 18 major distinct protein bands were evident, and the pattern looked identical to that in Coomassie blue-stained gels (Fig. 1). After blotting, the gels were stained with Coomassie blue and no remaining protein was observed, indicating that the transfer worked efficiently.

***M. leprae* antigens recognized by IgG antibodies in sera from patients with LL.** Lepromatous patients produce high titers of anti-*M. leprae* antibodies, mainly of IgG and IgM isotypes, which may be detected in their blood for many

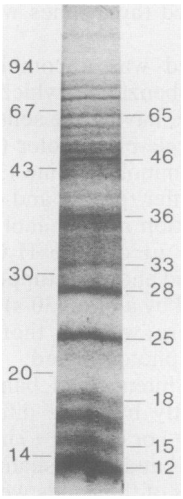

FIG. 1. SDS-PAGE of *M. leprae* sonic extract. *M. leprae* sonic extract CD99 (10 μg) was separated by SDS-PAGE and stained with Coomassie blue. The molecular masses of major protein bands are indicated on the right, with molecular mass standards (in kilodaltons) on the left.

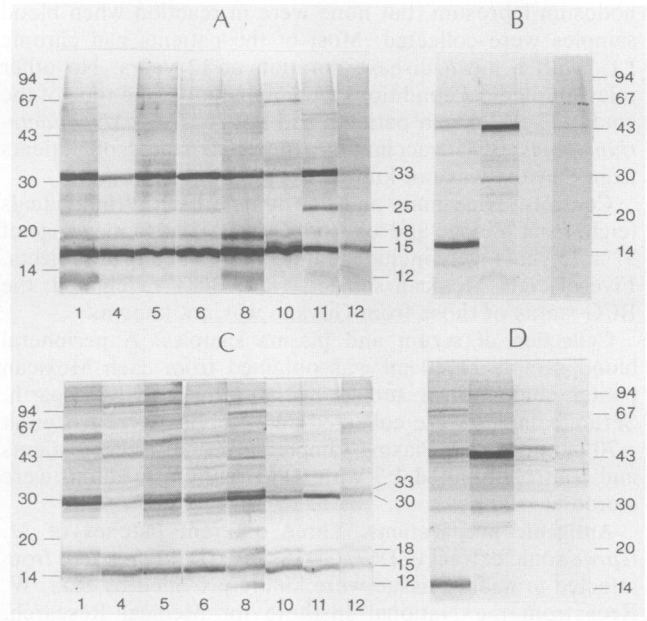

FIG. 2. Recognition of mycobacterial antigens by IgG antibodies from patients with LL and controls by Western blot analysis. Immunoblotting of *M. leprae* sonic extract and antigenic recognition profiles by sera from patients with LL (A) and Mexican controls which recognized antigenic bands (B). Immunoblots of *M. tuberculosis* soluble extract were probed with LL (C) and control (D) sera. For sera from patients, the lanes are numbered at the bottom with patient numbers. The same four controls were used in panels B and D. (Controls that showed no reactivity were not included.) Each panel of samples was run in a separate gel. The molecular masses (in kilodaltons) of the five major *M. leprae* antigens recognized are indicated.

years in spite of treatment. Since IgG antibodies are more specific and require T-cell cooperation for their production, we investigated the recognition of mycobacterial antigens by IgG class antibodies contained in the sera of these lepromatous patients. The pattern of recognition was the same whether whole LL serum or protein A affinity-purified IgG class antibodies from the same patient were used (data not shown).

All 20 samples from these LL patients contained IgG antibodies that recognized protein antigens in the *M. leprae* sonic extracts (Fig. 2A). Identical results were reproduced with three different batches of *M. leprae* sonic extract. Each sample recognized between 7 and 11 distinct bands on the blots. Five bands (33, 25, 18, 15, and 12 kDa) were seen most frequently and were more intense than other proteins. In Fig. 2A, results obtained with a selection of Mexican sera are illustrated in which binding to the five major antigens could be clearly observed. The results are summarized in Fig. 3.

To determine the nature of the antigens recognized, experiments with enzymatic treatment of *M. leprae* sonic extract were performed. After the sonic extract was digested with trypsin or *S. griseus* protease, most of the protein bands disappeared from a gel stained with Coomassie blue (data not shown). The carbohydrate running in the region of 30 to 36 kDa and stained with periodic acid-Schiff stain was unaffected by protease treatment. An identical gel was blotted and probed with an LL serum sample known to identify antigens of 33 and 15 kDa. The antigenic recognition of these proteins was lost after enzymatic treatment of the sonic extract (data not shown). This demonstrates that the 33- and 15-kDa antigens contain protein.

Samples from healthy donors. Samples from 10 of 14 control subjects showed no binding to *M. leprae* sonic extracts (data not shown). The other four showed binding, but to fewer bands and with less intensity than those from patients (Fig. 2B). It was concluded that most of the antibodies present in the sera of LL patients were due to anti-*M. leprae* activity, since both patients and controls shared a similar antigenic environment but only the samples from patients recognized *M. leprae* antigens with high intensity and frequency.

Murine monoclonal antibodies. To determine whether the antigens recognized by the LL samples corresponded to proteins to which murine monoclonal antibodies have been raised, five *M. leprae*-specific murine monoclonal antibodies (which recognized 65-, 36-, 28-, 18-, and 12-kDa proteins) were used in parallel with sera from patients (Fig. 4). The 33-, 25-, and 15-kDa antigens identified by sera were clearly distinct from the proteins identified by the murine monoclonal antibodies. However, the 18- and 12-kDa bands detected by sera were indistinguishable from those bound by the corresponding murine monoclonal antibodies under the conditions used here. The murine monoclonal antibody to the 65-kDa protein did not detect a distinct band in any of the three antigen preparations used; instead, a number of bands smaller than 65 kDa, probably degradation products, were observed.

***M. leprae* antigens recognized by IgM antibodies.** Lepromatous patients also produce high titers of IgM antibodies to *M. leprae*; in particular, IgM antibodies to phenolic glycolipid I are frequently detected in LL sera (18). To investigate recognition of *M. leprae* protein components by IgM antibodies, blots were probed with LL sera as described above, with an IgM-specific secondary antibody.

A higher background was observed than with the IgG antibodies. A number of distinct low-intensity bands were distinguishable when the serum was more concentrated, at a 1:50 dilution. As with IgG, most sera detected the 33-, 18-,

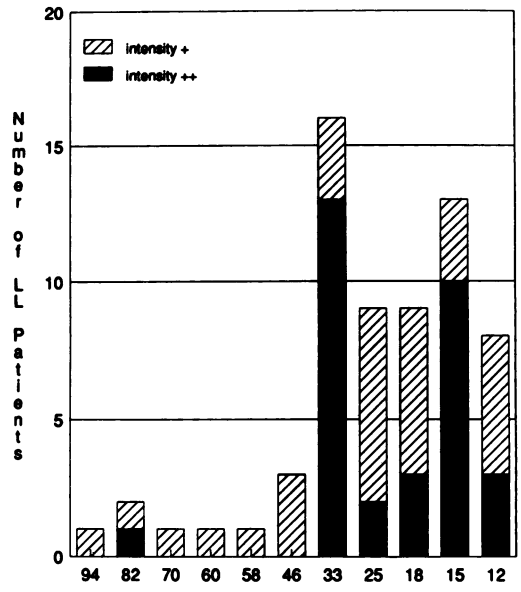

FIG. 3. Recognition of *M. leprae* protein antigens by IgG antibodies from 20 patients with LL. The hatched bars represent the number of patients with LL whose sera recognized each of 11 *M. leprae* antigens of the indicated molecular masses. The number of patients whose sera recognized antigens with high intensity is shown in black bars. The numbers on the bottom indicate molecular masses in kilodaltons.

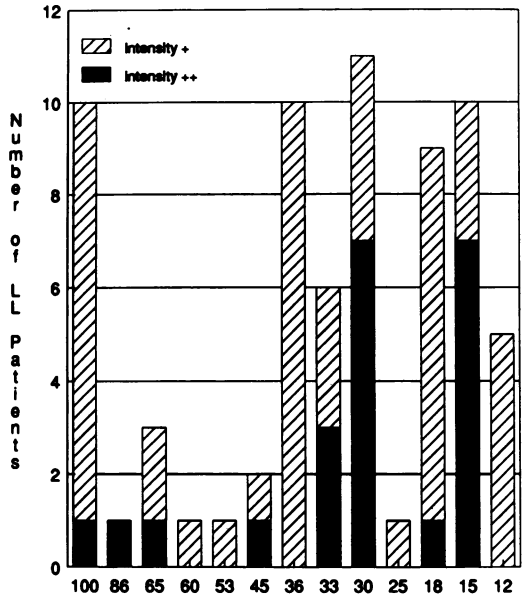

FIG. 5. Recognition of *M. tuberculosis* protein antigens by IgG antibodies from 11 patients with LL. The hatched bars represent the number of patients with LL whose sera recognized 13 *M. tuberculosis* antigens of the indicated molecular masses. The number of patients whose sera recognized antigens with high intensity is shown by black bars. The numbers on the bottom indicate molecular masses in kilodaltons.

and 15-kDa proteins. A number of higher-molecular-mass bands were also visible (data not shown).

Recognition of *M. tuberculosis* antigens by sera from patients with LL and controls. To investigate the cross-reactivity of anti-*M. leprae* antibodies in LL patients, 11 serum samples from patients and 4 from controls were tested with an *M. tuberculosis* soluble extract blotted onto nitrocellulose strips. IgG class antibodies in these 15 sera recognized several antigens of *M. tuberculosis*. Whereas homologous molecules may have different molecular masses in different mycobacterial species, a striking feature of the results obtained here was the similarity of recognition patterns for 33-, 18-, 15-, and 12-kDa proteins (Fig. 2C). The 25-kDa band present in *M. leprae* was, however, absent in *M. tuberculosis*. The results are summarized in Fig. 5.

Only the four control sera which gave positive signals on the *M. leprae* sonic extract were tested with the *M. tuberculosis* soluble extract. As before, they recognized fewer proteins than did sera from patients (Fig. 2D).

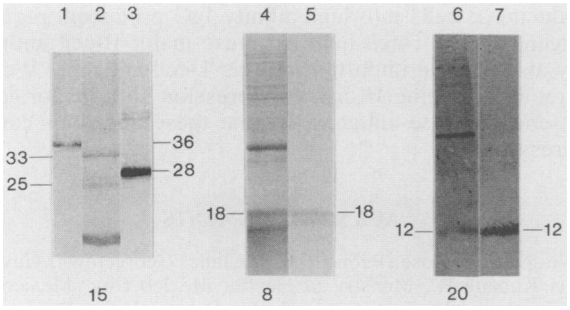

FIG. 4. Comparison of Western blotting profiles obtained with LL sera and *M. leprae*-specific murine monoclonal antibodies. *M. leprae* sonic extract was probed with selected LL sera (lanes 2, 4, and 6) and murine monoclonal antibodies (lanes 1, 3, 5, and 7). Lanes: 1, F47-9 (36 kDa); 3, B11-H (28 kDa); 5, L5 (18 kDa); 7, ML06 (12 kDa). The molecular masses of the bands recognized by murine monoclonal antibodies are indicated to the right of the lanes, whereas the figures to the left correspond to the antigens of 33, 25, 18, and 12 kDa recognized by LL sera. The numbers at the bottom are patient numbers.

DISCUSSION

In this report we describe five *M. leprae* protein antigens with molecular masses of 33, 25, 18, 15, and 12 kDa which were recognized by a high proportion of sera from patients with LL. The 33- and 15-kDa components were repeatedly recognized with the highest intensity. The patients came from areas in Mexico and Guyana endemic for tuberculosis and leprosy, and most had not received BCG vaccination. We did not find any relationship between the patterns of antigenic recognition and the following assessed clinical data of the patients: age, sex, duration of disease, length of treatment, bacterial index, and history of erythema nodosum leprosum. Most of the control sera showed no binding to the antigens.

Comparisons between previous studies and our own are difficult because of the variation in the techniques and sera used and because of slight differences in estimations of the molecular weights of proteins. The most similar study (8) used only three LL sera and estimated the major low-molecular-mass proteins to be 13.5 and 28 kDa. In general, though, the researchers found more reactivity to high-molecular-weight proteins than was evident in our experiments. The three patients in that study were all untreated, unlike our own.

Klatser et al. (13) concentrated on identification of *M. leprae*-specific proteins. After absorption with other myco-

bacterial extracts, bands of 12, 22, and 33 kDa were found in the sera of five LL patients. As with our study, all sera were from patients undergoing multiple-drug therapy. Britton et al. (4) looked at immunoprecipitation of *M. leprae* proteins by sera from untreated patients with leprosy. As with our study, and in contrast to the results of Ehrenberg and Gebre (8), the major antigens identified with LL sera were of low molecular mass (48, 36, 33, 27, 15, and 12 kDa).

Lipoarabinomannan (LAM), a component of many mycobacteria, including *M. leprae*, has previously been shown to migrate during SDS-PAGE to form a band with an apparent molecular mass of 30 to 38 kDa (10), raising the possibility that it is related to the 33-kDa antigen we describe here. Probably the protease-resistant 30- to 36-kDa band stained with Schiff reagent in our gels corresponds to this lipopolysaccharide antigen. It has been suggested (10) that the broad 33-kDa band described as glycoprotein by Chakrabarty et al. (6) is LAM, despite its protease sensitivity. However, Britton et al. (2) showed that protease treatment of their antigenic preparation did not affect the binding of LAM-specific murine monoclonal antibodies. More recently, they demonstrated that proteins of 33 and 36 kDa (comigrating with LAM) can be immunoprecipitated by LL sera (4). We have shown that the antibody reactivity in our blots, which occurred as a discrete band of 33 kDa, is directed to protein or glycoprotein antigenic determinants. We therefore conclude that the 33-kDa antigen we have described is not LAM.

The variability of the results reported from different laboratories can be explained in different ways. (i) The experimental details and sensitivity of detection may differ. (ii) Two studies looked at untreated patients (4, 8), and others looked at patients under treatment (13; this work). It might be expected that the destruction of bacilli caused by antibiotic treatment would lead to a different antibody response. However, when Klatser et al. (13) looked at the antigens bound by serum from a lepromatous patient in eight sequential samples after initiation of multidrug therapy, they observed a reduction after several months in the intensity by which some antigens were bound, but the overall pattern remained the same. In our study, duration of treatment did not seem to correlate with differences in response; a patient under treatment for 1 week showed a pattern similar to those of patients treated for a year or more. (iii) Different *M. leprae* preparations may contain different antigens, and therefore, even with the use of standardized techniques, variations occur. This study and the work done by Chakrabarty et al. (6) were performed with the standard sonic extract preparation of *M. leprae* provided by the WHO, whereas other authors have prepared their own sonic extracts (8, 13) by different protocols. (iv) Genetic differences among human populations may affect the immune response to *M. leprae*, determining the susceptibility to different clinical types of leprosy and, as a consequence, yielding different antibody profiles. The studies described above involved patients from Ethiopia (8), Nepal (4), Mexico, and Guyana (this study). Individual donors may also recognize different epitopes on the same antigens. In addition to this, the diversity in the epidemiological patterns of types of leprosy around the world, the distribution of other mycobacterial diseases like tuberculosis, and the occurrence of environmental mycobacteria, are relevant factors which may affect production of antibodies to these antigens. National health policies in relation to BCG vaccination are also important.

Murine monoclonal antibodies to a number of *M. leprae* proteins have been raised. How relevant the proteins which these murine antibodies recognize are to human disease is unclear, but they are often quoted as being immunodominant. We have demonstrated that three of the five major antigens described here (33, 25, and 15 kDa) are distinct from those recognized by murine monoclonal antibodies. The 18- and 12-kDa antigens detected may be identical to those bound by murine antibodies L5 and ML06, but this needs confirmation. The 65-kDa protein, which appears to be an immunodominant and highly cross-reactive antigen (5), was not detected as a single band of 65 kDa in our gels. However, murine monoclonal antibody L12 bound a series of bands smaller than 65 kDa, presumably because of breakdown of the protein. The same was true of all three batches of *M. leprae* sonic extract, suggesting that it may be necessary to include protease inhibitors in the preparation of the sonic extract.

In our study, antibodies present in sera from patients with leprosy detected antigens in the soluble extract of *M. tuberculosis*, showing the same pattern of reactivity to 33-, 18-, 15-, and 12-kDa proteins as with *M. leprae* sonic extract. This suggests that they are homologous proteins which cross-react between the two species. If these proteins were to be of use in serodiagnosis, a species-specific epitope would have to be identified. The 25-kDa *M. leprae* protein was detected by most sera from patients, but no homolog was seen in *M. tuberculosis*. This may, therefore, be a candidate for use in a serodiagnostic test. The 22-kDa band described by Klatser et al. (13) may correspond to the 25-kDa protein we describe, which would provide further evidence that it is *M. leprae* specific. The ideal antigen used for a diagnostic test should allow identification of persons with multibacillary and paucibacillary leprosy; although we used sera from lepromatous patients for this study, it would be interesting to see whether the 25-kDa protein antigen is also recognized by sera from persons with paucibacillary leprosy.

Although B-cell antigens may be of some use in serodiagnosis and may be relevant to the pathologic changes caused by the disease, it is the cellular arm of the immune response which is generally believed to be protective against *M. leprae*. It has been shown that mycobacterial antigens which are recognized serologically may carry different epitopes with relevance for cellular recognition and development of effector functions (15). Furthermore, maturation of IgM-producing B cells into high-affinity IgG producers requires antigen-specific T-cell help, so these major B-cell antigens may also play an important role as T-cell antigens. We are currently screening *M. leprae* expression libraries for genes that encode these antigens, so that these questions can be addressed.

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